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*Published in:*  
Methods in Gut Microbial Ecology for Ruminants

*Link to article, DOI:*  
[10.1007/1-4020-3791-0\\_13](https://doi.org/10.1007/1-4020-3791-0_13)

*Publication date:*  
2005

*Document Version*  
Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

*Citation (APA):*  
Aminov, R. (2005). 16S/18S ribosomal DNA clone library analysis of rumen microbial diversity. In *Methods in Gut Microbial Ecology for Ruminants* (pp. 163-174). Springer Netherlands. [https://doi.org/10.1007/1-4020-3791-0\\_13](https://doi.org/10.1007/1-4020-3791-0_13)

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## 5.1. 16S/18S ribosomal DNA clone library analysis of rumen microbial diversity

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### Introduction

The rumen contains a complex ecosystem where billions of bacteria, archaea, protozoa and fungi reside. This diverse microbiota is well adapted to live in the rumen and play an important role in the digestion of feed and nutrient supply to the host in the form of microbial protein and volatile fatty acids. It is estimated that the rumen microbial population consists of about  $10^6$  protozoa/ml,  $10^3$ – $10^7$  fungi/ml,  $10^{10}$  bacteria/ml, and  $10^9$  methanogens/ml [11]. To better understand the complex relationships in the rumen, it is necessary to gain an insight into the diversity of the rumen microbes and how the quantity and composition of rumen micro-organisms are altered by a number of different host factors such as age, genetics and diet.

In the past, the diversity of micro-organisms from the digestive tracts of domesticated ruminants has been identified by classical microbiological techniques [43]. However, given the fastidious growth requirements of rumen micro-organisms, it is reasonable to concede that the culture-dependent methods may select against some species, or taxonomic groups, leading researchers to underestimate the microbial diversity that is actually present in the rumen. In fact, it has been speculated that 90% of micro-organisms in nature have escaped traditional cultivation methods [35]. Therefore, a major challenge in microbial ecology has been to assess the diversity and structure of natural microbial communities.

The field of molecular biology has advanced with many innovative technological breakthroughs. The ability to extract and to isolate high-molecular weight DNA from rumen digesta, PCR amplify genes from specific microbial groups and obtain gene sequence data is now a routine event. The small subunit ribosomal RNA (SSU-rRNA) gene, called 16S in prokaryotes and 18S in eukaryotes, is the most widely used molecular marker to presumptively identify morphologically indistinguishable species, to infer their phylogenetic relationships, and to elucidate microbial diversity. Furthermore, the retrieved SSU-rDNA sequence information lays a background for the development of other molecular detection techniques such as FISH, FISH-FC, DNA microarrays and quantitative real-time PCR techniques. The application of molecular techniques has already revealed the enormous wealth of microbial diversity and putative novel species in the rumen [15, 17, 21, 22, 30–32, 34, 39, 40, 45]. In

this Chapter, the main steps of SSU-rDNA library generation and analysis will be presented and discussed in detail.

### **Overview of experimental approach**

In general, construction of SSU-rDNA libraries is started from obtaining a fresh sample of the rumen contents from fistulated domestic animals or wild ruminants and fractionation if desirable (rumen fluid, rumen solids and protozoal fractions). At this stage, the sample can be frozen at  $-80^{\circ}\text{C}$  for further analysis. Then the total DNA is isolated using either commercially available kits, bead-beating technique, freezing–thawing method or enzymatic lysis. Obviously, this total DNA preparation contains chromosomal DNA of all microbiota, including bacteria, archaea, fungi and protozoa, as well as the host's DNA from sloughed-off epithelial cells. To retrieve specific SSU-rDNA sequences from this mix, PCR amplification with primers that are specific for bacteria, archaea, fungi or protozoa is used. The number of cycles needs to be maintained low (i.e. 10–12 cycles) to avoid preferential amplification of certain templates and/or chimeric molecule formation [3, 42].

Commercially manufactured cloning kits are available to clone the PCR amplified 16S/18S genes from your target group into specially designed plasmids. Positive clones (i.e. white colonies) are randomly selected and checked for the presence of expected size inserts by PCR amplification, this time using the vector primers that surround the insert. These recombinant clones now represent your library and the next step is to analyse them by sequencing.

In a randomly generated library, some clones are closely related and therefore redundant in terms of uncovering the whole diversity. Obviously, an initial selection step before proceeding to the complete sequencing is helpful to reduce the sequencing work while allowing the maximum diversity coverage in a library. This can be done by partial sequencing of PCR templates that were generated in the positive clone screening procedure of the previous step. For this, the PCR products are sequenced with one of the primers used for library production and the sequences are analysed for similarity. Sequences with similarity values  $\geq 97\%$  are considered to belong to the same operational taxonomic unit (OTU) and sequencing can be limited to the one representative OTU. This molecular species definition, OTU, is based on correlation between a DNA reassociation value and SSU-rDNA homology. In a polyphasic approach, the 'species' nomination includes bacteria that share a DNA reassociation value of about 70% [37], and this value corresponds to similarities of 97% and higher at the level of SSU-rDNA sequence [28]. For ecological community structure analysis, OTUs are considered as molecular species, while the number of sequences within an OTU represents the population of a given species. Complete sequencing of each representative OTU is done with several sequencing primers complementary to conserved regions of the SSU-rDNA molecule.

Alternatively, the taxonomic groups can be defined on the basis of restriction fragment length polymorphisms (RFLP), also called ribotypes. For this, instead of

sequencing, the PCR fragments are digested with a combination of frequent-cutting restriction enzymes according to the manufacturers specifications. Digested DNA is separated on 3–4% molecular screening agarose gel run at 100 V for at least 2 h. Gels are visualized with UV illumination and documented either by Polaroid photography or by a gel documentation system. The ribotypes are grouped according to their unique pattern and when possible, compared to expected ribotypes for known sequences (of the same length) from the same taxonomic group of interest. Otherwise, 5–10 representatives from each unique ribotype are sequenced for identification or confirmation of presumptive identification based upon expected ribotype pattern.

Further library analysis requires a computer with a set of programs for sequence analysis and Internet access. First sequences from each template are assembled and checked on-line for integrity using the CHIMERA\_CHECK program at the Ribosomal Database Project II (<http://rdp.cme.msu.edu/cgis/chimera.cgi?su=SSU>). This is an important step for cleaning-up a library by eliminating PCR-generated artefacts, such as chimeric sequences. The sequences that passed quality control are aligned together with the type strains and similar sequences from databases. This alignment now can be used for phylogenetic reconstruction using various methods such as neighbour-joining, maximum parsimony or maximum likelihood followed by statistical analysis, such as bootstrapping.

Based on the number of species retrieved (OTUs) and population numbers within the species (number of clones forming an OTU), the ecosystem parameters, such as library coverage, general diversity indices, evenness and species richness, can be elucidated.

### **Procedures, equipment and reagents**

For each animal, fresh rumen content samples should be used and transported on ice in a tightly closed tube to prevent further microbial activity. In a lab, rumen content can be fractionated if desired and immediately frozen and stored at  $-80^{\circ}\text{C}$ . Methods to collect, fractionate and extract DNA from rumen digesta and PCR amplification protocols are suggested in Chapter 3.

There are several methods of total DNA isolation, including commercially available kits (e.g. Qiagen QIamp<sup>®</sup> DNA Stool Mini Kit, Qiagen, Valencia, CA), bead-beating technique, freezing–thawing method or enzymatic lysis. In our experience of bacterial library construction, the bead-beating method, which is also implemented in some kits, results in the maximum recovery of diversity, while the enzymatic lysis method generates libraries biased toward over-representation of Gram-negative bacteria belonging to the CFB phylum (Tajima et al., unpublished observations). Despite the higher probability of chimera formation with template DNA produced by bead-beating, it remains the method of choice for production of PCR-generated SSU-rDNA libraries. This is, in part, because no high-molecular weight DNA is required for this application and the probability of cell disintegration is independent of the cell wall structure.

There are some concerns about library generation by PCR, in that, the number of cycles must be kept as small as possible. It has been shown for the human gut community that the 25-cycle rDNA library displays reduced diversity than the 10-cycle library [3]. The smaller number of cycles imposes strict requirements to DNA quality; it can be additionally purified by gel-filtration, cesium chloride (CsCl) gradient centrifugation or agarose electrophoresis, if PCR-inhibiting substances are suspected in DNA preparations. Another point of the library quality issues is the annealing temperature during PCR. A significant increase in number of OTUs retrieved can be achieved by lowering the annealing temperature [12].

It is also important to mention that most commercial cloning kits use the A-overhang artefact that is generated by *Taq* polymerase and PCR products are ligated into plasmids designed with a T-overhang. Therefore, on the last PCR cycle, the primer extension should be between 7 and 10 min long to ensure completion of the A-overhangs. This will also improve your cloning efficiency. Because there are many commercial cloning kits available, such as the TA cloning kit (Invitrogen), pGEM-T cloning kit (Promega), the Sure Clone Ligation Kit (Pharmacia), etc., it is important to follow the manufacturers instructions. PCR primers used to amplify bacterial, archaeal, protozoal and fungal SSU-rDNA are shown in Table 1.

### *Cloning*

1. After PCR, the mix can be used directly in the cloning reaction with TOPO TA cloning kit (Invitrogen, CA, USA). This kit uses topoisomerase-mediated incorporation of PCR amplicons into the T-overhang vector pCR4 and all cloning procedures can be done within 2 h.
2. To ensure colonies are well spread on the 1.2% LBAXI agar plates (LB agar containing Ampicillin, Xgal and IPTG) (Sigma; Appendix VI), spread three different volumes (120  $\mu$ , 80  $\mu$  and 50  $\mu$ l) using a hockey stick and incubate at 37°C for 14–16 h.

If using TOP10 cells, no IPTG is required for blue/white screening.

(*Note:* A bacterium without an insert in the plasmid forms blue colonies (i.e. negative) and a bacterium with an insert in the plasmid forms white colonies in the presence of Xgal (i.e. positive) [25].)

3. Remove the plates with the transformed colonies and store at 4°C for several hours to increase the intensity of blue (i.e. negative) colonies.

### *Clone confirmation*

1. Use the blunt end of an autoclaved toothpick to gently touch the centre of a single white colony and make three streaks 1–2 cm long onto a new 1.2% LBAXI agar plate. Repeat this step using a new toothpick for each white colony, and then incubate at 37°C for 12–16 h.
2. Remove LBAXI agar plates and check triple streaks for blue colouration. Disregard blue streaks and number only those sets of triple streaks that are white.

Table 1. Primers used for library construction and analysis

Primer ID	Primer sequence	References
<i>Archaea</i>		
025eF	5'-CTG GTT GAT CCT GCC AG	[1]
1492R	5'-GGT TAC CTT GTT ACG ACT	[1]
D30	5'-ATT CCG GTT GAT CCT GC	[2]
D33	5'-TCG CGC CTG CGC CCC GT	[2]
Met83F	5'-ACK GCT CAG TAA CAC	[44]
Met86F	5'-GCT CAG TAA CAC GTG G	[44]
Met1340R	5'-CGG TGT GTG CAA GGA G	[44]
Ar1000	5'-AGTCAGGCAACGAGCGAGA	[38]
Ar1500	5'-GGTTACCTTGTACGACTT	[38]
<i>Bacteria</i>		
27f	5' AGA GTT TGA TCM TGG CTC AG	[16]
519f	5' CAG CMG CCG CGG TAA TWC	[16]
519r	5' GWA TTT TAC CGC GGC KG C TG	[16]
926r	5' CCG TCA ATT CMT TTR AGT TT	[16]
926f	5' AAA CTY AAA KGA ATT GAC GG	[16]
1492r	5' TAC GGY TAC CTT GTT ACG ACT T	[16]
1525r	5' AAG GAG GTG WTC CAR CC	[16]
<i>Eukarya</i>		
Medlin B	5'-TGA TCC TTC TGC AGG TTC ACC TAC	[19]
<i>Fungi</i>		
nu-SSU-0817f	5' TTA GCA TGG AAT AAT RRA ATA GGA	[4]
nu-SSU-1196r	5' TCT GGA CCT GGT GAG TTT CC	[4]
nu-SSU-1536r	5' ATT GCA ATG CYC TAT CCC CA	[4]
<i>Protozoa</i>		
P-SSU-342F	5'-CTT TCG ATG GTA GTG TAT TGG ACT AC	[13]

- For each group of white streaks, use the blunt end of an autoclaved toothpick to collect cells from one of the three streaks. Pick one blue streak and use it as your negative control.
- Stick toothpick and cells into 15  $\mu$ l of distilled H<sub>2</sub>O in a 0.6 ml microfuge tube. Twist toothpick in the distilled H<sub>2</sub>O to dislodge the bacterial cells (water should turn cloudy) and discard the toothpick into a biohazardous waste autoclave bag. Do not leave toothpick in tube or it will absorb the solution.
- Add 15  $\mu$ l of 2 $\times$  plasmid cracking buffer and vortex. Bacterial cells are lysed when the solution turns clear.
- Add 30  $\mu$ l of distilled H<sub>2</sub>O to the solution and then add 5  $\mu$ l of 6 $\times$  dye.

To check for plasmids containing the correct size insert, load 30  $\mu$ l of each tube onto a 1.0% agarose gel and run for 1 h at 100 V. First load a DNA molecular marker and then the sample from the negative control (i.e. blue streak containing plasmid only).

(Note: The DNA will be supercoiled, so it will migrate faster than linear DNA. To determine the approximate size of your positive clone on the gel, take the size of your plasmid and add the size of your PCR product and then look for a band at approximately 60% of your calculation. For example, if your plasmid is 2.9 kb

and your insert is 1.3 kb insert (4.2 kb total), then 60% of 4.2 kb would be the supercoiled size.)

7. Identify and label all samples of the correct molecular weight (i.e. those larger than the negative control).
8. Aseptically remove one of the two remaining triple streaks from each positive clone using an inoculation loop or an autoclaved toothpick and inoculate test tubes containing 10 ml of LB broth and Ampicillin.
9. Agitate tubes overnight on a platform shaker at 37°C.  
(*Note:* The remaining streak can be used as a backup or to make glycerol stocks for storage.)

#### *Alternative rapid protocol for clone confirmation*

If you have access to a genomics facility, recombinant colonies are picked up using an automated colony picker into the 96- or 384-well format microtitre plates. The cells are grown overnight in 2× LB or terrific broth and archived with 20% glycerol or 7% DMSO at −80°C. The liquid handling robot is then used for PCR amplification using 1 µl of cell suspension as a template and vector primers (M13 or T3 + T7). One microlitre of the resulting PCR is used for sequencing PCR. If no high-throughput facility is available, follow the protocol given below.

1. Prepare PCR master mix with BIOTAQ Red polymerase (Bioline) and M13 or T3 and T7 primers.
2. Dispense 15 µl aliquots into a 96-well PCR plate on ice using a 12-channel pipette.
3. Use a sterile plastic toothpick to gently touch the centre of a single white colony and dip it into the PCR mix for 1–2 s. Then inoculate the biomass remaining on a toothpick to a corresponding grid on a 96-well microtitre plate with ×2 LB or terrific broth. Repeat the step using a new toothpick for each white colony.
4. Pick a blue colony as a negative control.
5. Seal the plate and run PCR with initial DNA denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 10 s, 55°C for 10 s and 72°C for 30 s, then the final elongation step at 72°C for 10 min and hold at 4°C.
6. In the meantime, prepare a 1% agarose gel in TBE with a GelStar fluorescent dye (BMC).
7. Load 5 µl aliquots into the gel directly from the PCR plate using a 12-channel pipette. No need to add loading buffer. Run phoresis at 100 V for 1 h.
8. After electrophoresis, document the gel with the GelDoc system. ‘Empty’ clones will produce a small PCR product, 150–190 bp of length, migrating together with the negative control, while recombinants will produce larger amplicons (ca. 1.5 kb if bacterial libraries were produced with 27f and 1525r or 27f and 1492r primer pairs).
9. The remaining PCR product (step 7) can be stored at 4°C for sequencing with a single primer (e.g. with 27f primer in a bacterial library that was produced with 27f and 1492r primers) to define the number of OTUs in your library.

10. Incubate the plate from step 3 at 37°C for 12–16 h with gentle agitation and archive it at –80°C after adding glycerol (20% final concentration) or DMSO (7%).

### *Plasmid miniprep*

Plasmids are harvested and purified from the overnight cultures using plasmid minipreps such as the Flexi-Prep 100 Kit (Qiagen) and GenElute™ Plasmid Miniprep kit (Sigma). The plasmid insert is then re-amplified by PCR using the vector-specific primer, usually M13 or T3 and T7.

### *Determination of OTUs by ribotyping*

1. Frequent-cutting restriction endonucleases are used, in accordance with manufacturer's specifications, to digest the amplified SSU-rDNA sequence.
2. Digested fragments with a 100 bp DNA molecular weight marker (to determine the size of the bands) are then separated by gel electrophoresis performed at 100 V for 2 h on high-resolution 3–4% molecular screening agarose gel.
3. Gels are visualized with UV illumination and ribotypes documented either by Polaroid photography or by a gel documentation system.
4. Ribotypes are grouped according to their unique pattern.
5. At least 5–10 representatives from each unique ribotype are sequenced for identification, ideally in both directions using a set of primers covering the whole amplicon.

### *Determination of OTUs by sequencing*

1. Perform sequencing PCR with a 1 µl template from a positive PCR reaction (Chapter 3) and an internal sequencing primer (e.g. 338F for bacterial libraries, or Met448F for methanogen libraries [44]).

(Note: If PCR was performed with a large excess of primers and dNTPs, they may interfere with the subsequent sequencing PCR and need to be removed by ExoSAP-IT (Amersham Biosciences). For this, add 1 µl of the ExoSAP-IT enzyme mix to 5 µl of PCR product and incubate at 37°C for 15 min, followed by enzymes inactivation at 80°C for 15 min. Use 1 µl of it in cycle sequencing (e.g. with BigDye v.3.1, Applied Biosystems, or other chemistries.)

2. Clean up the sequences and generate a DNA similarity matrix. Reads with more than 97% similarity represent a single OTU and 1–2 clones from each OTU are submitted to complete sequencing by a combination of primers covering the whole amplicon on both strands (e.g. in the case of 27r and 1492r-generated bacterial libraries, these are 27f, 338f, 338r, 907r, 926f and 1492r; or in the case of Met83F or Met86F and Met1340R-generated methanogen libraries these are Met86F, Met448F, Met448R, Met1027F, Met1027R and Met1340R [44]).



*Bioinformatics*

Phylogenetic classification is an evolutionary hypothesis of a historical sequence of speciation events that suggests that species or groups of species classified together are more closely related genealogically to each other than they are to any other taxa [10, 41]. This system is based on the principle of homology and uses *a priori* arguments based on comparisons to an outgroup. Theoretically, any taxonomic unit not in the ingroup can be used as the outgroup. However, ideally you should use the sister group for comparisons [18, 27, 29, 36].

1. Assemble several sequencing reads of each OTU using Sequencher or AutoAssembler (PE-Applied Biosystem). Clean up from vector sequences and edit the sequence if necessary.
2. Check the integrity of your sequence using the CHIMERA\_CHECK program at the Ribosomal Database Project II ([http://rdp.cme.msu.edu/cgis/chimera.cgi?su = SSU](http://rdp.cme.msu.edu/cgis/chimera.cgi?su=SSU)).
3. Align new sequences against existing sequences for your taxonomic group using sequence alignment software such as Dedicated Comparative Sequence Editor (DCSE) [23] or CLUSTALX [33] (available at <http://www-igbmc.ustrasbg.fr/BioInfo/ClustalX/Top.html>). For phylogenetically coherent sequences, remember to use sequences from closely related species for your outgroup. If the library is generated with primers targeting the larger order taxa such as the *Bacteria* domain, use the deep-branching sequences of *Thermotoga* and *Aquifex*.
4. Check the alignment quality and edit if necessary (now the alignment is ready for phylogenetic analysis). A phylogenetic inference package such as PHYLIP (v. 3.51C) [6] can be used to calculate the sequence similarity and evolutionary distances between pairs of nucleotide sequences using a distance model such as the Kimura two-parameter model [14].
5. Construct a distance-matrix tree using either the Fitch and Margoliash least squares method [7] or the neighbour-joining method [24].
6. To determine the stability of particular groups of taxa, bootstrap resamples [5] the data 1000 times. When phylogenetic trees are constructed by distance-matrix methods, the horizontal length of the branches produced from the analysis indicates the amount of genetic difference between the organisms, in relation to each other on the tree.
7. Visualize and edit the tree using TreeView, NJ plot, or MacClade program.

*The Shannon index values*

The Shannon index illustrates the difference in diversity between SSU-rDNA clone libraries. The Shannon index ( $H$ ) of general diversity can be calculated for each clone library using the following equation [20].

$$H = - \sum \left( \frac{n_i}{N} \right) \times \log \left( \frac{n_i}{N} \right)$$

where  $n_i$  = importance value for each species

$N$  = total of importance values

The Shannon index is calculated as follows: the total importance value is the total number of clones identified in that sample. Thus, the importance value for the individual clone was the number of times it was identified in the sample. The SSU-rDNA clone library with the greatest diversity has the highest Shannon index value.

### *Rarefaction analysis*

The diversity of your clone libraries can be estimated by rarefaction analysis [9] using the Analytic Rarefaction v.1.3 program (S.M. Holland, University of Georgia), which is freely available on the Web ([www.uga.edu/strata/software](http://www.uga.edu/strata/software)).

### *Library coverage*

The library coverage value is calculated according to Good [8] using the formula  $[1 - (n/N)]$ , where  $n$  is the number of OTUs represented only by a single clone and  $N$  is the total number of clones in the library. The more unique OTUs in the library, the lower the diversity coverage. This approach also helps to test whether two libraries (e.g. from different diets) are significantly different and can be calculated with the LIBSHUFF program [26] available on the Web ([www.arches.uga.edu/~whitman/libshuff.html](http://www.arches.uga.edu/~whitman/libshuff.html)).

## **Solutions**

*1.0 M Tris-HCl, pH 8.0*

*0.5 M Na-EDTA, pH 8.0*

*0.5 M Na-EDTA, pH 7.5*

*5 M NaCl*

### *Xgal/IPTG (5 : 1) solution*

- 5-Bromo-4-chloro-3-indolyl galactopyranoside (Xgal) 100 mg
- Isopropyl thio- $\beta$ -D-galactoside (IPTG) 200 mg
- Dimethylformamide 5 ml
- Autoclaved distilled H<sub>2</sub>O 1 ml

Dissolve 100 mg of Xgal in 5 ml of dimethylformamide (20 mg/ml).

Dissolve 200 mg of IPTG in 1.0 ml of autoclaved distilled H<sub>2</sub>O (200 mg/ml)

Mix 5 ml of Xgal solution with 1 ml of IPTG and store at  $-20^{\circ}\text{C}$ .

### *Luria Bertini (LB) broth*

- NaCl 5.0 g
- Yeast extract 2.5 g
- Bacto tryptone 5.0 g

Mix ingredients and bring to 500 ml with distilled H<sub>2</sub>O, then aliquot 10 ml into 40 test tubes. Use 100 ml for 100× Ampicillin solution (see below). Autoclave test tubes, and when cool using sterile techniques add 100 µl of the 100× Ampicillin solution to each tube. Store tubes at 4°C.

*100× Ampicillin solution*

- LB broth 100 ml
- Ampicillin 1.0 g

Dissolve 1.0 g of Ampicillin into 100 ml of LB broth. No need to autoclave as this is 100× recommended strength. Keep frozen at −20°C.

*1.2% LB–Ampicillin–Xgal–IPTG (LBAXI) agar*

- NaCl 10.0 g
- Yeast extract 5.0 g
- Bacto tryptone 10.0 g
- Agar 12.0 g
- Xgal : IPTG (5 : 1) solution 1.0 ml
- 100× Ampicillin solution 10.0 ml (or 100 mg Ampicillin)

Mix all dry ingredients and bring to 1 l with distilled H<sub>2</sub>O and autoclave. When solution is cool enough to touch against wrist, add Ampicillin and 1 ml of Xgal : IPTG (5 : 1) solution. Swirl the contents and pour into (30–40) Petri dishes.

*2× Plasmid Cracking Buffer*

- 10 M NaOH 0.5 ml
- 0.5 M Na-EDTA pH 8.0 1.0 ml
- 20% (v/v) SDS 2.5 ml
- Glycerol 5.0 ml
- Distilled H<sub>2</sub>O 41.0 ml

Mix solution and store at room temperature. No need to autoclave.

*6× Dye*

- Bromophenol blue (final concentration 0.3%) 0.3 g
- Xylene Cyanol FF (final concentration 0.3%) 0.3 g
- 0.5 M Na-EDTA (pH 7.5) (final concentration 10.0 mM) 2.0 ml
- 40% (w/v) Glucose solution 98.0 ml

Mix solution and store at −20°C. No need to autoclave.

## Other reagents and equipment

DNA isolation kits, PCR kits, primers, pipettes and tips, electrophoresis unit, thermocycler, UV transilluminator, spectrophotometer (A<sub>260</sub> nm capable), restriction enzymes, DNA size markers, agarose (multipurpose and molecular screening), centrifuge, incubator, water bath, capillary sequencer, computer and software for sequence alignment and phylogenetic analysis.

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